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Ethanol Project
Strain Development Team

Summary of Technical Achievements or Results

The Strain Development Team (SDT) successfully developed a new, single *Zymomonas* biocatalyst that can convert xylose and arabinose (along with glucose) to ethanol. Seven genes (xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-p 4-epimerase, transketolase, and transaldolase), which encode the enzymes needed to convert xylose and arabinose to common intermediates of *Zymomonas*' central glycolytic pathway, were simultaneously introduced into *Zymomonas* under the control of strong promoters that direct their expression, even in the presence of glucose.

The newly engineered strain can grow on and ferment xylose, arabinose, or glucose as sole carbon sources to ethanol and ferments a mixture of 1% glucose, 2% xylose, and 2% arabinose to ethanol at about 90% of the theoretical yield at 30°C without pH control. Further fermentation performance under various pH, temperatures, and high sugar concentrations, will be evaluated.

This new *Zymomonas* strain appears stable, and will be most useful for lignocellulosic feedstocks (such as switchgrass, wheat straw, corn cobs, corn fiber, and spent grains) that contain significant quantities of both pentose sugars. Further, introducing additional exogenous genes into *Zymomonas* did not dramatically affect the overall metabolic burden, which suggests that direct microbial conversion work may be fruitful in the future.

General Technical/Scientific Progress

A series of mixed-culture cofermentations was run in which the xylose- and arabinose-fermenting *Zymomonas* strains were combined in the presence of glucose, xylose, and/or arabinose.

The results clearly demonstrated a hierarchy of sugar utilization in which glucose was preferred over xylose, and xylose was preferred over arabinose. Also, arabinose did not affect xylose transport in the presence of glucose, but arabinose uptake was compromised in the presence of xylose. This hierarchy was also observed in the newly developed single xylose/arabinose-fermenting *Zymomonas* strain.

A succession of fermentations was performed to evaluate the ability of recombinant *Zymomonas* to grow on and effectively ferment sugars at the high process concentrations in feedstreams generated by potential customers such as Arkenol or the CRA/NCGA. The process yields of recombinant *Zymomonas* were similar to those of wild-type *Zymomonas* at up to 15% glucose (with no adaptation). Also, the recombinant *Zymomonas* performed well in xylose concentrations as high as 10%.

Using a method newly developed to detect sugar alcohols by high-performance anion-exchange chromatography with pulsed amperometric detection, we confirmed that xylitol and arabitol were formed in addition to glycerol, lactic acid, and acetic acid during mixed sugar fermentations using *Zymomonas* strains. This will enable us to better understand the metabolism of recombinant *Zymomonas* and design strategies to eliminate by-product formation by inactivating the branch pathways if necessary.

Subcontract research on "Investigate Pentose Sugar Transport in *Zymomonas*" during the past year by T. Conway of Ohio State University showed that xylose is transported exclusively via the glucose-facilitated transporter Glf. Xylose transport rate is subject to carbon source-dependent and growth phase-dependent regulation. Xylose transport is strongly inhibited by glucose and less inhibited by L-arabinose. Glf has a much higher affinity for glucose as compared to xylose (about threefold higher Km toward xylose compared to glucose). Mutation of the native *Zymomonas* transporter Glf will be pursued to improve xylose transport.

Scientific Papers Submitted

A manuscript entitled, "Development of an Arabinose-Fermenting *Zymomonas mobilis* by Metabolic Pathway Engineering," was submitted to *J. Appl. Environ. Microbiol.* on September 24.

EI gene into a plasmid (pUC 19) suitable for performing the mutagenic reactions in *Escherichia coli* (Epicurian Blue/XL1). Using this system, the EI-CAT was finally expressed at sufficient levels for biochemical testing (0.5-1 mg/L). We are now cloning all 13 mutant EI enzymes discussed above and preparing for comparative kinetic testing.

Scientific Publications, Presentations, and Other Activities

General Presentations/Travel

M. Himmel and S. Thomas attended a 2-day strategy session with Drs. Wilson and Karplus at Cornell University to define the highest benefit plan for site-directed modifications to improve this important enzyme.

Scientific Meetings: Posters Presented or Recently Accepted for Presentation

The following oral paper and poster session paper were presented at the 18th Symposium on Biotechnology for Fuels and Chemicals held in Gatlinburg, TN:

- "Cellulase Superfolds: Diversity of Structure and Convergence of Function"
- "A Membrane-Reactor Saccharification Assay to Evaluate the Performance of Cellulases and Substrate Pretreatments under Simulated SSF Conditions."

Technology Transfer

M. Himmel and NREL Legal issued a draft materials transfer agreement to Genencor International for testing the *A. cellulolyticus* EI endoglucanase.

S. Thomas collaborated with S. Austin-Phillips at the University of Wisconsin regarding her interest in developing a plant expression system for the *A. cellulolyticus* EI endoglucanase and *T. reesei* CBH I. A materials transfer agreement is in preparation.

Scientific Journals

The following papers are in press:

"A Membrane-Reactor Saccharification Assay to Evaluate the Performance of Cellulases under Simulated SSF Conditions," by J.O. Baker, T.B. Vinzant, C.I. Ehrman, W.S. Adney, and M.E. Himmel, *Appl. Biochem. Biotechnol.* 1997.

"Polysaccharide Hydrolase Folds: Diversity of Structure and Convergence of Function," by M.E. Himmel, J. Sakon, J.O. Baker, W.S. Adney, P.A. Karplus, and S.R. Thomas, *Appl. Biochem. Biotechnol.* 1997.

"Crystal Structure of Thermostable Family 5 Endocellulase EI from *Acidothermus cellulolyticus* in Complex with Cellotetraose," by J. Sakon, W. Adney, M. Himmel, S. Thomas, and P. Karplus, *Biochemistry* 1996, *35*, 10648–10660.

The following manuscript has been submitted for consideration:

"Advanced Bioethanol Production Technologies: A Perspective," M. Himmel, W. Adney, J. Baker, R. Elander, J. McMillan, R. Nieves, J. Sheehan, S. Thomas, T. Vinzant, M. Zhang, *Fuels and Chemicals from Biomass*; ACS Symposium Series Book, 1997.

Project Operation

The subcontract to Cornell University (Dr. Karplus) entitled, "Provide X-Ray Structures of Cellulases," was initiated and is on schedule.

A consultant agreement with Dr. Wilson at Cornell was finalized and initiated. This agreement permits Dr. Wilson to actively support and advise the Biofuels program on cellulose biochemistry-related matters.

M. Himmel and S. Thomas enrolled in a 2-day training course at Molecular Simulations Inc., in San Diego, CA. This formal exposure to molecular structure analysis has proven valuable to our work for DOE Biofuels.

the ammonia recycled percolation process, that should be investigated further.

Screening experiments were conducted to evaluate the yield of sugars from the pretreatment of softwoods. Douglas Fir "forest litter" was pretreated under a range of temperatures from 140°C to 210°C, using hot-water or dilute-acid catalyst for 10–30 min. The most promising results were achieved at 210°C, using dilute acid for 30 min. All the hemicellulosic sugars and 45% of the cellulose were hydrolyzed.

A series of experiments was conducted in conjunction with the Industrial Technologies Group to determine the potential of zeolites catalysts to detoxify prehydrolyzate liquors. Using the Mobil Zeolite HZM-5, several screening experiments were conducted to evaluate the effect of temperature and flow rate on the detoxification of the pretreatment liquor. More than 38% reduction in the toxicity of the liquors could be achieved at 140°C operating conditions. Further reductions could be achieved by optimizing the retention time, temperature, and catalyst particle size. Further studies using the zeolites will include upgrading lignin for higher value coproduct credits.

Technical Presentations/Posters

Pretreatment Team

R. Torget gave a presentation to the International Energy Agency (IDEA) on modeling pretreatment processes at the 18th Symposium on Biotechnology for Fuels and Chemicals in Gatlinburg, TN.

Patents or ROIs filed

Pretreatment Team

The patent entitled, "Hydrolysis and Fractionation of Lignocellulose," has been filed by the NREL patent office.

General Presentations/Outreach

Pretreatment Team

R. Elander and R. Torget gave a general presentation to Sunds Corp. about developing a prototype prehydrolysis reactor to evaluate material handling and mass transfer of solids and liquor in a countercurrent configuration. A purchase order was submitted for such a reactor on September 30.

of 50% (v/v), a cellulase enzyme loading of 12 filter paper units per gram cellulose, and an SSCF processing time of 7 days. The system achieved 54% conver- sion of all potentially available biomass sugar (total sugars) entering SSCF; a control SSCF run under similar conditions using 50% (v/v) conditioned hydrolysate but employing Sigmacell cellulose and a commercial cellulase achieved 65% conversion of total sugars to ethanol. These experiments demonstrated that at modest enzyme loading the integrated SSCF process performs at or very close to the milestone performance target of 60% of theoretical yield based on total sugars entering SSCF.

We subsequently examined the process qualifier experimental results and developed 14 proposals for improving the performance of the integrated sawdust-to-ethanol process to achieve the ethanol project's MYTP year 2000 performance objectives. These proposals were then ranked using cost/impact analysis coupled with a rule-based multi-attribute decision making methodology. This process indicated that several proposals have excellent near-term potential to significantly reduce bioethanol production cost. Plans to execute them are currently being finalized.

General Technical/Scientific Progress

Several important pretreatment-related capabilities were improved during the reporting period, including improvements to the system that feeds the pilot-scale Sunds hydrolyzer, to the Sunds hydrolyzer reactor itself, as well as substantially completing installation and testing a flexible acid impregnation system (the high-pressure acid impregnator [HPAI], see below); other pretreatment-related capabilities improvements are described in the process qualifier milestone report. Substantial progress was also made in establishing the capability to perform integrated system testing at the mini-pilot scale. These accomplishments are described below.

Improved Sunds Hydrolyzer Feed System

The feed system was substantially improved. A new dust containment system was installed for the feedhopper. In addition, the discharge chute from the feedhopper to the first weighbelt in the feed system was refabricated to eliminate the constricting tapered wall construction of the original installation. The new chute design has straight vertical walls, which greatly reduce the potential for biomass bridging. A heavy-duty gear box was also installed to upgrade power transmission to the bridge breakers for the feedhopper. Cross bars were welded to the tines of the bridge breakers to increase their effectiveness.

The discharge chute from the first weighbelt was refabricated to eliminate the many sharp corners and constrictions that were present in the original installation. This modification greatly reduces the occurrence of biomass bridging at the second bridge point in the feed system to the Sunds hydrolyzer.

Finally, a vibratory screen was installed on the feedhopper to screen out large rocks and tramp metal in the feed. Two powerful magnets were installed in the discharge chute from the first weighbelt to trap tramp metal. In combination, these modifications will ensure a much more trouble-free system for feeding the Sunds hydrolyzer. These improvements in the operability of the system that feeds the Sunds have already benefited the SWAN CRADA work, which used the biomass feeding system (but not the Sunds hydrolyzer). During FY 1997, some industrial partners and the EPD are expected to benefit from the improved operability of the system that feeds the Sunds (as well as in the operability of the Sunds itself—see below).

Improved Sunds Hydrolyzer

An greatly oversized steam flow meter for the Sunds hydrolyzer was replaced with a correctly sized flow meter, which also required modifying the steam piping to the Sunds hydrolyzer. Steam traps were added to the Sunds hydrolyzer steam supply to eliminate condensate carryover into the steam flow meter and reduce noise during data acquisition (during experimental work). The original installation lacked these steam traps and, as a result, there was considerable noise in the steam flow rate measurements.

A vent and vent condenser were also added to the Sunds hydrolyzer. The vent removes the noncon-

process data, including the gas mass flow and composition data, thereby enabling us to generate mass balances over the entire system.

We initiated a comprehensive series of tests on the ISTB system to validate its acceptability for conducting experiments that involve the recombinant *Z. mobilis*. This requires approval by NREL's Institutional Biosafety Committee (IBC) to operate the system at the BL-1 LS biosafety level. This approval requires that the capabilities of the system be validated to contain recombinant microorganisms and to properly inactivate the microorganisms to be investigated under worst-case processing conditions expected in the planned experiments. Experiments targeted at obtaining IBC approval are under way.

We have already demonstrated the ability to maintain asepsis throughout the fermentors and the complex biomass recirculation loops. Pasteurization and sterilization of liquid broths also have been demonstrated using cultures of the parent microbe from which the recombinant *Zymomonas* was produced.

We also demonstrated the ability to maintain asepsis during processing of highly concentrated solid slurries (~20% w/w solids). The next steps are to demonstrate pasteurization and/or sterilization of 20% (w/w) biomass slurries that contain a live culture of the parent *Zymomonas* strain. A final report that documents system validation then must be submitted to NREL's IBC and approval obtained to commence planned integrated performance testing at the mini-pilot ISTB scale. We expect to obtain approval to operate the ISTB at BL-1 LS during the first quarter of the next reporting period.

Scientific Publications, Presentations, and Other Activities

Patents or ROIs Filed

Two ROIs were filed by the EPD team during the reporting period. Both describe processes for conditioning pretreated materials to enable better fermentation performance:

- The first is entitled, "A solvent extraction process for conditioning lignocellulosic biomass pre-hydrolysis and complete hydrolysis products to increase fermentation efficiency," and was invented by C. Hatzis, T.K. Hayward, and D. Glassner.

- The second is entitled, "An ion-exchange adsorption process for removing specific inhibitory compounds from lignocellulosic biomass prehydrolysis and complete hydrolysis products to increase fermentation efficiency," and was invented by C. Hatzis, K. Evans, and D. Glassner.

Scientific Meetings: Papers and Posters Presented or Accepted for Presentation

J. McMillan and presented a paper, "Bioethanol Production: Status and Prospects," at the World Renewable Energy Congress held June 16–21 in Denver, CO.

The following posters were presented at the Eighteenth Symposium on Biotechnology for Fuels and Chemicals held May 6–9 in Gatlinburg, TN:

- "Sugars from Biomass as a Raw Material for Renewable Chemicals," by C. Hatzis, S. Schmidt, T.K. Hayward, N. Padukone, and R. Wooley.

- "Strategic Approaches to a Balanced R&D Portfolio in the Renewable Chemical Industry," by C. Hatzis and N. Padukone.

- "Optimization of Seed Production for a Simultaneous Saccharification and Cofermentation Process Using Recombinant *Zymomonas*," by H. Lawford and J. Rousseau of the University of Toronto and J. McMillan of NREL.

- "Evaluation of PTMSP Membranes for Enhanced Ethanol Removal from Fermentation by Pervaporation," by M. Myers, S. Schmidt, N. Padukone, J. McMillan, and S. Kelley.

A paper entitled, "Technoeconomic and Kinetic Modeling—Foundational Tools for Guiding Pretreatment Research at NREL (1980–1996)," written by C. Hatzis, R. Torget, V. Putsche, and R. Elander,

Coors/NREL CRADA

Phase 1 of the Coors/NREL CRADA was completed July 31. The C-milestone, "Coors CRADA Phase 2 Go/No-Go Decision," was delayed 1 month (until July 31) to allow additional testing to better determine the value of the fermentation residual solids. The Coors Team recommends proceeding with Phase 2, which will include more detailed pretreatment and fermentation laboratory work by NREL and business plan development and site-specific preliminary engineering by Coors.

PDU Team

Members of the PDU team spent April through June finishing the Task 5 run under the Amoco CRADA. Following a short downtime to repair equipment, Gridley Phase 1 operations began and continued into September. This work included modifying the PDU feed system to accomodate rice straw. Those members of the PDU team not on Gridley either moved to other projects or began longer-term maintenance projects in the PDU, including flooring rework and instrumentation calibration. These continued in September.

Acknowledgments

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Amoco CRADA

The Amoco CRADA, initiated in 1991, is nearing completion of the third of four phases. The objective of Phase 3 is to provide sufficient information at pilot scale to justify construction of a demonstration facility for converting biomass to ethanol. There were three major components to the Phase 3 work:

- A laboratory program to evaluate fermenting microorganisms
- A series of experiments in the NREL process development unit (PDU)
- Process design and economic modeling.

The laboratory program and PDU experiments are complete. The PDU experiments included two successful 6-week continuous runs in which the PDU operated 24 hours a day, 7 days a week. The final conceptual process design and economic evaluation are currently under way to determine the feasibility of a demonstration plant and continuation of the CRADA to Phase 4.

was presented at the IEA Workshop held immediately following the 18th Symposium.

The following posters were presented at the Ninth Annual Colorado Biotechnology Symposium held October 8, 1996, at Colorado State University in Fort Collins, CO:

- "Investigation of the Effects of Long-term Storage on the Chemical Composition and Biological Toxicity of Acidic Hardwood Hydrolyzate," written by N. Dowe, W. Keutzer, and C. Hatzis.

- "Rapid Determination of Cellulose in Pretreated Hardwood by Modification of ANKOM semi-automated Forage Fiber Analyses," written by F. Keller and Q. Nguyen.

- "Characterization of Two Xylose-Fermenting *Zymomonas mobilis* Strains for Continuous Ethanol Production from a Glucose-Xylose Mixture," written by A. Mohagheghi, S. Toon, M. Newman, M. Zhang, and J. McMillan.

densable gases in the biomass fed to the Sunds hydrolyzer. This continuous purging of the noncondensable gases has allowed the Sunds to be operated nearer the desired experimental temperature. In addition, experiments have shown that with a 5% bleed rate approximately 8% of the toxic furfural formed during pretreatment is removed in the vent gases. Some acetic acid is also removed.

Installed HPAI

The HPAI is designed to impregnate wood chips or sawdust with dilute acid at conditions above atmospheric pressure and temperature. It was designed to accommodate operating conditions typically used in commercial chemical impregnators, and can be used as a percolation reactor for biomass pretreatment or for extracting hemicellulose and lignin from pretreated lignocellulosic materials. We expect that the HPAI, because of its flexibility, will ultimately provide data useful for designing and scaling up pretreatment equipment. It is currently being used to acid impregnate materials to be pretreated in the NREL digester, the pretreatment reactor developed to scale down the Sunds hydrolyzer (see "Process Qualifier" report for more details on developing and testing the NREL digester).

In addition to EPD, planned FY 1997 work associated with the softwood development and Coors CRADA projects is expected to use the HPAI/NREL digester to enable efficient exploration and optimization of high-solids pretreatment conditions. The HPAI consists of two 40-L feed vessels, one 15-L impregnation reactor, and one 40-L product tank. The tank and piping parts in contact with acidic liquors are made of Hastelloy-C to resist corrosion. In a typical acid impregnation procedure, sawdust is weighed into a cylindrical canister with slotted screens at both ends. The filled canister is then placed in the impregnator. Low-pressure steam then purges air from the sawdust. Dilute-acid solution, preheated in one of the feed vessels, is circulated through the sawdust. If necessary, nitrogen gas is used to equalize the pressure between the two vessels. After 1–2 h of recirculating the acid, the acidic liquor is drained from the canister back into the feed vessel. The acid-impregnated sawdust is removed for further processing (dewatering and then pretreatment). If the HPAI is to be used to extract the biomass, the liquid solution (water, dilute acid, or dilute alkali solution) can be either recirculated through the bed of biomass (sawdust or pretreated material) or pumped through in a single pass.

Mechanically Completed and Qualified Mini-Pilot Integrated Stirred Tank Bioreactor System

The mini-pilot scale integrated stirred tank bioreactor (ISTB) system was installed, and initial qualification experiments are complete. The mini-ISTB system is now more than three-quarters operationally qualified and validated. Current plans for FY 1997 activities include a significant amount of experimental work that involves bioprocessing at high solids concentrations that require the use of the ISTB system. Other teams required to demonstrate bioconversion at high solids concentrations are also likely to use the mini-pilot system in FY 1997 and beyond.

We demonstrated the ability of the ISTB system to homogeneously mix and transfer biomass slurries between bioreactors using total solids (biomass) loadings above 20% (w/w). Slurry transfer rates are controlled via high-sensitivity load cells. The supervisory control system (see below) uses the load cell measurement signals in combination with the process set point targets to calculate and dose nutrient and enzyme solutions in proportions appropriate for the amounts of biomass fed to each bioreactor.

The ISTB data acquisition and control system (DACS) is designed to enable the bioreactors to be controlled either locally (manually) or remotely (via computer). We have established and demonstrated manual control of the biomass feed rate and are now working to implement the ability to control the biomass feed rate continuously so we can carry out continuous processing in the ISTB system. So far, we have established the ability to operate the system in either batch or fed-batch mode.

The ISTB DACS uses G2 supervisory control software. It has been developed to the point at which all available bioreactor data are being logged and processed by the G2 software, except for gas mass spectrometer and gas mass flow rate data. When complete, the ISTB DACS system will acquire all

Ethanol Project
Ethanol Process Development

Summary of Technical Achievements or Results

Ethanol Process Development (EPD) team activities focused on core technology process development. The highlight accomplishments are completion of two August 1996 C-level milestones for the integrated process development project. The EPD team also developed new pretreatment and integrated bioprocessing capabilities that will support industrial partners and core technology process development projects (Coors CRADA, softwood development, and others). Capabilities enhancements include:

- Improving the operability of the pilot-scale Sunds hydrolyzer pretreatment reactor
- Establishing a scale-down capability for the Sunds hydrolyzer based on the use of a novel acid impregnator in combination with the NREL digester pretreatment reactor
- Substantially completing the mini-pilot scale system that will be used for integrated process demonstration as the process is scaled up further. The following sections describe each achievement in greater detail.

Two milestones are complete:

- "Evaluate enzymatic hydrolysis and plan for future actions," involved reviewing cellulase production literature and evaluating the feasibility of enzyme production and enzymatic hydrolysis in an enzyme-based biomass-to-ethanol process using NREL's ASPEN/IPE technoeconomic process model. Results of this analysis showed that enzymatic hydrolysis is economically feasible if the enzyme yield on substrate and the volumetric rate of enzyme production meet the upper third of values reported in the literature. Results of this analysis show that the economics of enzymatic hydrolysis are particularly sensitive to the yield of cellulase enzyme on substrate but less sensitive to the enzyme production rate. Another important finding is that the cost of enzyme production may be affected by scale-up, as economies of scale are limited by the agitation and aeration requirements for cellulase enzyme production. These findings have been used to develop a plan for experimentally determining the key cellulase production performance metrics (i.e., yield and production rate). This plan is currently being implemented.

- "Process qualifier milestone"involved demonstrating a minimum level of performance for an integrated sawdust-to-ethanol process that incorporates dilute-acid pretreatment and NREL's xylose-fermenting *Z. mobilis* to convert conditioned pretreatment whole slurry to ethanol in a simultaneous saccharification and fermentation (SSCF) process configuration.

  To effectively integrate pretreatment and SSCF, methods of conditioning (detoxifying) pretreatment hydrolysate developed during the last reporting period were further developed and then scaled up to enable researchers to prepare enough material for integrated performance testing. In parallel with this activity, SSCF and inoculum production processes were characterized using the adapted strain of the xylose-fermenting *Zymomonas*. A set of integrated SSCF demonstration experiments was ultimately performed with a system that incorporates the following major process components:

  - Cocurrent dilute-acid pretreatment of yellow poplar sawdust in the Sunds pilot scale pretreatment reactor
  - A new liquid-liquid extraction-based hydrolysate conditioning process
  - *Trichoderma reesei* MTC-a-13 cellulase enzyme produced using PYP solids
  - The *Zymomonas* strain adapted to perform better in yellow poplar hydrolysate.

  The performance of the integrated SSCF process was tested using a hydrolysate concentration

Ethanol Project
Pretreatment

Summary of Technical Achievements of Results

The major focus of the pretreatment group is to continue testing the complete hydrolysis process, which will allow the hemicellulose fraction of hardwood poplar sawdust (and a significant fraction of the cellulose) to be hydrolyzed. It will use a countercurrent reaction mode to reduce the formation of degradation products and maximize sugar yields. Converting the cellulose and hemicellulose fractions could radically improve the general economics of the ethanol process. Key issues addressed are the actual pretreatment yields, concept validation, detoxification of prehydrolysis liquor, and the large-scale reactor design.

Work continues to develop the following:

- Countercurrent operation of total hydrolysis process to achieve high sugar yields with little or no cellulase addition
- Developing the shrinking bed process to maximize the sugar concentration of the pretreatment liquor
- Using engineering based models such as ASPEN to determine the effect of alternative pretreatments, various pretreatment options, and hot-water pretreatment developments
- Examine the effect of zeolite treatment of the hydrolysis to improve detoxification.

General Technical/Scientific Progress

Pretreatment Team

A C-level milestone entitled, "Seek to achieve 80% yield of glucose equivalents with acceptable xylose yield equivalents in multistage dilute acid process with economic means of producing acceptable liquor volumes," was completed on May 31. The results of the milestone suggested that the dilute acid complete hydrolysis process could provide near quantitative sugar yields and high sugar concentrations without using an expensive cellulase unit operation.

The central idea behind the process is the continuous shrinking bed reactor design (CSBR). This allows higher linear velocities of liquid hydrolysis medium by shrinking the biomass bed as the hydrolysis reaction proceeds, but still maintaining a constant volumetric flow rate. The total hydrolysis of biomass using very dilute acid (0.07 wt %) and usisg the CSBR concept is significant in that it may eliminate or severely reduce the concentration of cellulase needed and the associated cost of cellulase production to the fermentation process.

The liquors from the hot-water preextraction of lignins from poplar sawdust were analyzed for their effect on short-term fermentation assays. The results indicated that the liquors are not toxic to the microorganisms. Solids have not been tested for any decrease in toxicity after further pretreatment. An expanded series of experiments has been conducted to evaluate the effect of retention time, particle size, and flow rate on the hot-water pretreatment of poplar saw dust. The results show that with a retention time of 2–3 min, at 200°C and flow rates of 500mL/min, more than 85% of the xylan was solubilized. However, only 60% of the feed xylan could be accounted for after the treatment. Thus, 40% of this sugar was lost to some degradation products. Reducing the particle size from 4mm to 1 mm did not increase solubilization of xylose or cellulose.

A C-level milestone, "Complete rank ordering of alternate pretreatment technologies," was submitted on September 30. A methodology had been developed to evaluate alternative pretreatment methods developed under various subcontracts. A cost/yield index was developed using the key capital and operating costs to evaluate the effectiveness of various pretreatment approaches. The results showed that dilute sulfuric acid had the highest ranking. None of the alkaline-based approaches offered a competitive advantage. However, there were some unique aspects of the other pretreatment approaches, such as

Ethanol Project
Enzyme Technology Team

Summary of Technical Achievements or Results

Our goal is to improve the performance (increase the specific activity) of cellulases that act on pretreated biomass used for bioethanol production. We are focusing on the highly active *Acidothermus cellulolyticus* EI endoglucanase, which works with a high degree of synergism with fungal exoglucanases, including *Trichoderma reesei* CBH I. To accomplish this task, we recently evaluated the chemical nature of pretreated biomass and established that dilute-acid pretreated hardwood tissue develops distinctly non-native surface chemistry. Thus, naturally occurring cellulase systems, such as the *T. reesei* complex, are unlikely to function ideally on this substrate. Changes in biomass include a redistribution of lignin following exposure to temperatures of about 160°C and an increase in the acidic nature of the biomass surface, which results from dilute-acid hydrolysis. These observations are new, and profoundly affect cellulase biochemistry and effectiveness.

When used in combination with *T. reesei* CBH I in diafiltration saccharification assays, the isolated catalytic domain of *A. cellulolyticus* EI is twice as effective as the intact enzyme (also used with CBH I) in the saccharification of dilute-acid-pretreated yellow poplar (PYP). Because the isolated catalytic domain (EI-CAT) does not display this advantage over the intact enzyme in the saccharification of pure microcrystalline cellulose (Sigmacell-20), the superiority of EI-CAT in the saccharification of PYP can be apparently be attributed to its ability to operate more successfully in the presence of the lignin component of the pretreated biomass substrate.

The new diafiltration-saccharification protocol has been used to test the performance of commercial cellulases and NREL-grown *T. reesei* preparations against PYP. Enzyme loadings for these tests were kept constant at 20 FPU/g cellulose and the temperature was 37°C to simulate current SSF conditions. This work clearly showed that *T. reesei* cellulase preparations grown and induced on PYP were superior in hydrolyzing those same substrates to *T. reesei* preparations grown and induced on soluble sugars (i.e., lactose). This finding has important implications on process design options and cellulase production.

General Technical/Scientific Progress

To improve the cost effectiveness of the EI endoglucanase, we are targeting improvements in its specific activity because a twofold improvement in specific activity should result in a twofold reduction in process requirement for this enzyme. Improvements in thermal stability are also desirable because they contribute to general process tolerance. Recent x-ray structural studies of EI have shown that it belongs to glycosyl hydrolase family 5. We have been able to take advantage of the work published from other laboratories on other family 5 enzymes to develop our EI strategy. For example, modifying most critical, highly conserved amino acid residues in the active sites of cellulases probably does not improve activity on cellodextrins; however, other residues (those that affect substrate surface/enzyme interaction) may contribute substantially to enzyme action.

Following this novel approach, we targeted two surface residues that define the contact path of a cellulose chain as it binds to the enzyme. Our strategy is to moderate the strength of the enzyme binding to the substrate to increase the reaction rate. This is being accomplished in EI by replacing residues tryptophan 42 (W042) and tyrosine 82 (Y082) with alanine (A), glycine (G), glutamic acid (E), glutamine (Q), and arginine (R) residues by site-directed mutagenesis (SDM). Another strategy is to increase the catalytic rate constant directly by decreasing the strength of the cellobiose binding leaving group from the distal end of the active site cleft. This is being accomplished by replacing tyrosine 245 (Y245) with A, E, Q, and R. These three hydrophobic residues are thought to provide platforms for binding sugar residues in the cellulose substrate chain.

After evaluating the Promega Altered States II SDM system, we adopted the Stratagene QuickChange SDM system and successfully cloned the truncated

Scientific Publications, Presentations, and Other Activities

Patents or ROIs Filed

An MRI/NREL patent, "Recombinant *Zymomonas* for Pentose Fermentation," was published as U.S. patent #5,514,583.

Two MRI/NREL patent applications have been filed and received first PTO actions:

- IR #95-26, "Recombinant *Zymomonas* for Pentose Fermentation"
- IR #95-27, "Pentose Fermentation by Recombinant *Zymomonas*"

A new ROI, IR #96-51, "Development of a Single *Zymomonas mobilis* Strain for Xylose and Arabinose Fermentation," has been filed with the NREL Legal Office.

Technology Transfer

Materials transfer agreements have been signed with the following companies:

- Michigan Biotechnology Institute for using our patented *Zymomonas* strains in a waste sludge ethanol plant
- Arkenol Energy Corporation for using our patented *Zymomonas* strains in its concentrated acid/bagasse ethanol pilot plant
- Unisearch Ltd. for using our patented *Zymomonas* strains

Approximately four other materials transfer agreements and licensing agreements for using our patented *Zymomonas* strains are in various stages of development.

Five members of the Corn Refiners and National Corn Growers Associations visited NREL to view a demonstration and evaluate our *Zymomonas* technology. In particular, these key ethanol producers were interested in its unique ability to ferment the glucose, xylose, and arabinose sugars in pretreated corn fiber. The SDT and Biofuels Program received a favorable response from this visit, and a statement of work was submitted to the CRA/NCGA, seeking additional funding to supplement and expedite our ongoing programmatic work.

Publications, Posters, and Presentations at Scientific Meetings

M. Zhang presented a poster entitled, "Metabolic Engineering of Pentose Metabolism Pathways in *Zymomonas mobilis* for Ethanol Production," at the Recombinant DNA Biotechnology: Focus on Metabolic Engineering meeting held in Danvers, MA, October 6–11.

A. Mohagheghi presented a poster entitled, "Characterization of Two Xylose-fermenting *Zymomonas mobilis* Strains for Continuous Ethanol Production from a Glucose-Xylose Mixture," at the CIRB meetings in Ft. Collins, CO, on October 8.

M. Zhang presented a poster entitled, "Development of an Arabinose-Fermenting *Zymomonas mobilis* by Metabolic Pathway Engineering," at the 18th Symposium on Biotechnology for Fuels and Chemicals in Gatlinburg, TN, May 6–9.

M. Finkelstein gave a talk entitled, "Ethanol Conversions with Genetically Engineered *Zymomonas mobilis*," at the World Renewable Energy Congress in Denver, CO, on June 19.

M. Finkelstein gave a talk entitled, "Advances in Biocatalyst Development for Conversion of Corn Fiber to Ethanol," at the Corn Utilization Conference in St. Louis, MO, on June 4.

Scientific Papers Accepted

A chapter entitled, "Biocatalyst Development for Bioethanol Production from Hydrolysates," written by S. Picataggio and M. Zhang, was published in the *Handbook on Bioethanol: Production and Utilization*, edited by C.E. Wyman, 1996.